Oberst et al. Supplementary Material

Supplementary Figure Legends

Figure S1: a. Cell death (Annexin V staining) of SV40 immortalized MEF from Capase-$8^{flox/flox}$:RosaCreER embryos. Caspase-8 was deleted in MEF by addition of 4-hydroxytamoxifen as indicated. Cells were treated with TNF for 3 hours as indicated in the presence of Annexin V-FITC, and bright field and fluorescent images were taken and merged. **b.** Overt normality of 15 week old littermate $RIPK3^{-/-}$:$Caspase\text{-}8^{+/-}$ and $RIPK3^{-/-}$:$Caspase\text{-}8^{-/-}$ mice. **c.** Weight vs. age of $RIPK3^{-/-}$ mice with the indicated caspase-8 genetic status. Logarithmic best-fit lines for each genotype are also shown. **d.** Western blot analysis of tissues taken from 15 week old mice of the indicated genotype. Note that the RIPK3 antibody used produced a faster-migrating aspecifically immunoreactive band in some tissues that was present irrespective of RIPK3 status. **e**. Cell death (Annexin V positivity) of thymocytes from mice of the indicated genotypes, treated with the indicated death inducing agents, with or without the caspase inhibitor qVD. All treatments were for 8 hours, except anti-Fas, which was used for 24 hours in combination with 0.25μg/ml cycloheximide. **f.** Whole livers from littermate animals of the indicated genotypes, taken 3 hours after Jo2 injection. **g.** 10X and 40X magnified sections of livers from mice of the indicated genotypes, prepared 3 hours after Jo2 injection and stained with hematoxylin and eosin (H&E). **h.** Serum levels of alanine aminotransferase (ALT; Error bars are s.e.m., n=9 each genotype) and aspartate aminotransferase (AST; Error bars are s.e.m, n=10 $casp8^{-/-}$, n=6 $casp8^{+/-}$) for mice of the indicated genotype immediately before or 3 hours after Jo2 injection.

Figure S2: a. Lymphoid organs removed from 4 week old littermate mice of the indicated genotypes. LN is Lymph Node. Scale bar is 1cm. **b.** Proliferation of CD3/CD28 activated splenic T cells of the indicated genotypes. Cells were stained with carboxyfluorescein succinimidyl ester (CFSE) and proliferation was assessed at the indicated timepoints. **c.** Relative Vβ6 and Vβ8 T-cell populations from mice of the indicated genetic status following treatment with the bacterial superantigen *staphylococcus* enterotoxin B (SEB). Error bars are s.d., n=3 animals per genotype **d.** FACS analysis of cells of the indicated tissues taken from 15 week old littermate $RIPK3^{-/-}$ animals of the indicated caspase-8 genetic status, stained with anti-CD3 and anti-B220.

Figure S3: a. Catalytic activity of a recombinant fusion protein consisting of the FKBP inducible-dimerization domain fused to the catalytic domains of caspase-8 (FKBP-$Caspase\text{-}8^{WT}$), or a similar protein bearing prohibitive mutations at the aspartate cleavage sites between the large and small subunits(FKBP-$Caspase\text{-}8^{DA}$). This protein was treated with the kosmotropic salt sodium citrate, or with the homodimerizer AP20187 as previously described, and activity was measured using the fluorogenic substrate IETD-AFC. ND indicates none detected. Error bars=s.d., n=3. **b.** Western blot analysis of MEF described in **3b,** transfected with a scramble siRNA, or with one of two siRNAs targeting FLIP. **c**. Western blot analysis of SVEC 4-10 cells stably expressing a scrambled shRNA or a shRNA specific for RIPK3, then transfected with scrambled siRNAs or siRNAs specific for caspase-8 or FLIP as indicated. Data presented are representative of similar results obtained with either of 2 siRNAs targeting caspase-8 or FLIP. **d.** Western blot analysis of two 3T3 murine fibroblast lines, either RIPK3 deficient (NIH) or RIPK3 expressing (SA). These cells were transfected with scrambled siRNAs or siRNAs specific for caspase-8 or FLIP as indicated. Data presented are representative of similar results obtained with either of 2 siRNAs targeting caspase-8 or FLIP. **e,f.** Cell death (PI uptake) of RIPK3 deficient (NIH, **e**) or RIPK3 expressing (SA, **f**) 3T3 cells transfected with siRNAs specific to caspase-8 or FLIP as indicated, then treated with TNF as indicated for 24 hours. Graph represents mean of two separate experiments, error bars show range**. g.** Western blot analysis of L929 cells stably expressing an shRNA targeting RIPK3, then

transfected with siRNAs specific to caspase-8 or FLIP as indicated. Data presented are representative of similar results obtained with either of 2 siRNAs targeting caspase-8 or FLIP. L929 cells expressing a scrambled shRNA or an shRNA specific to RIPK3 were also analyzed for RIPK3 expression to confirm reduction in protein level.

Figure S4: a, b. Cell death (PI uptake) of 3T3-SA cells stably expressing an shRNA construct specific for RIPK3 (**a**), or the same cells also stably expressing BCL-XL-GFP (**b**). These cells were transfected with siRNAs specific to caspase-8 or FLIP as indicated, then treated with TNF or TNF+Necrostatin-1 as indicated for 24 hours. Error bars=s.d., n=3. **c.** Western blot analysis of 3T3-SA (RIPK3-knockdown) cells stably expressing vector or Bcl-XL, then transfected with an siRNA specific to FLIP and treated with TNF for 8 hours as indicated. RIPK3 knockdown cells were used to minimize necrotic death and allow collection of lysates. **d.** Whole cell lysates from Bcl-XL expressing 3T3-SA cells transfected with siRNAs and treated with TNF as indicated were resolved by western blot using the indicated antibodies, prior to FADD immunoprecipitation.

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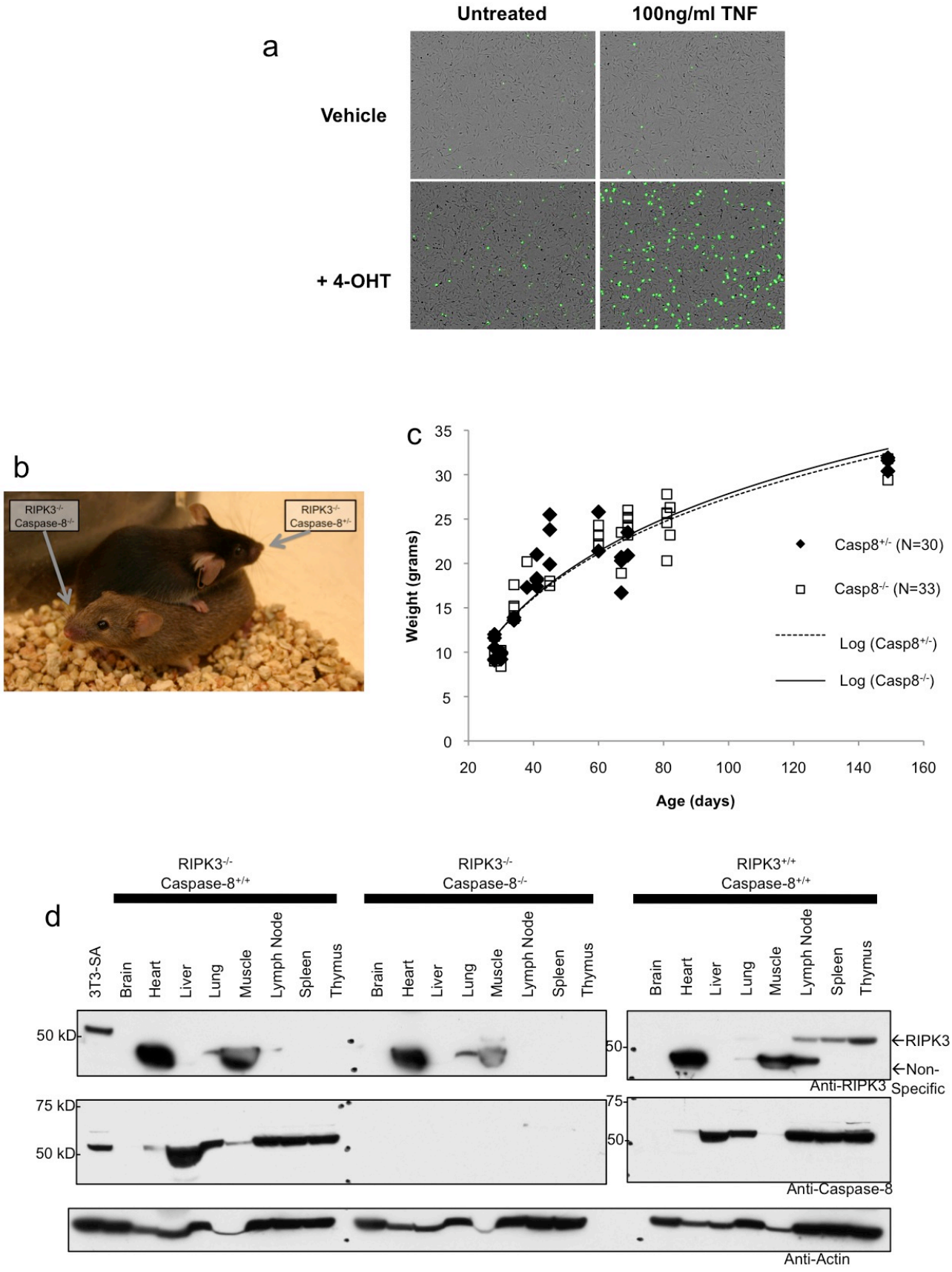
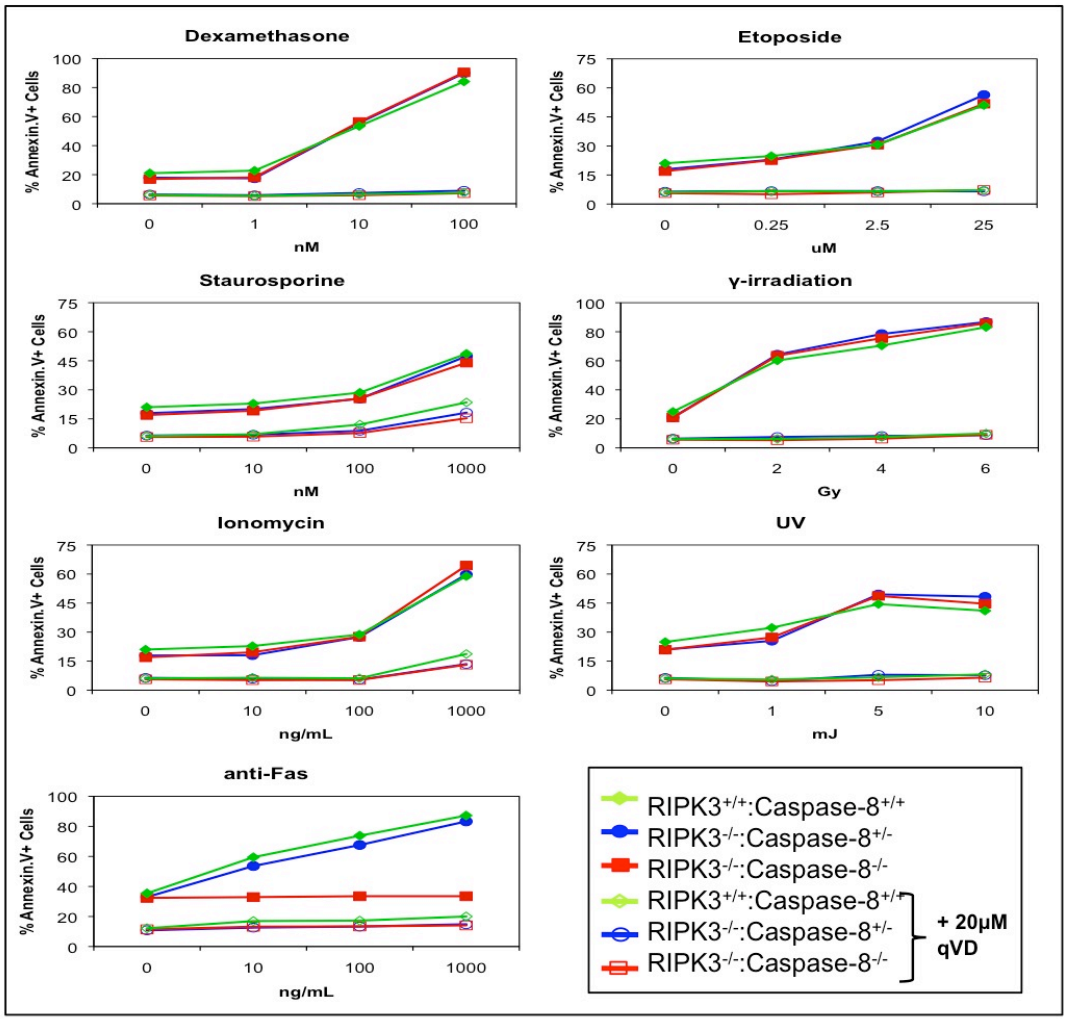
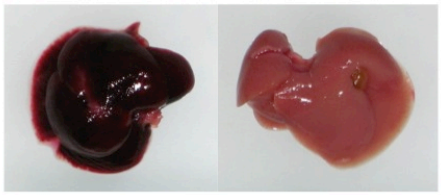
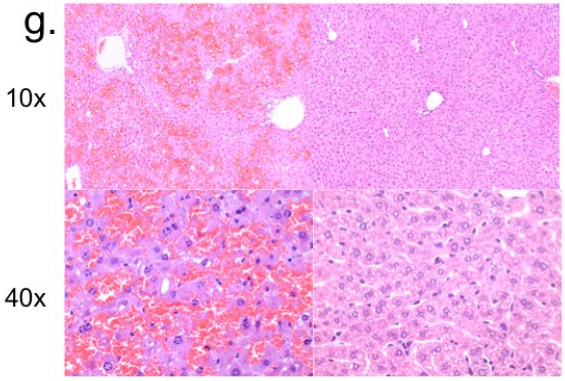
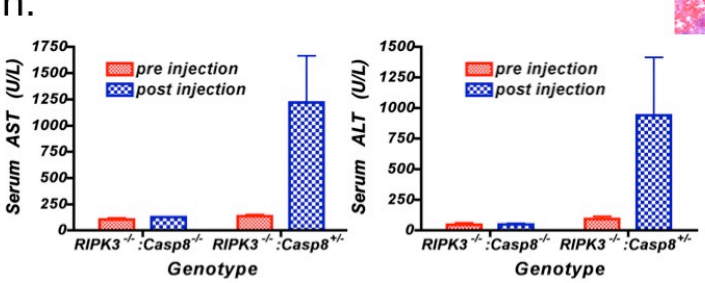


Oberst et al. Figure S1 (cont.)

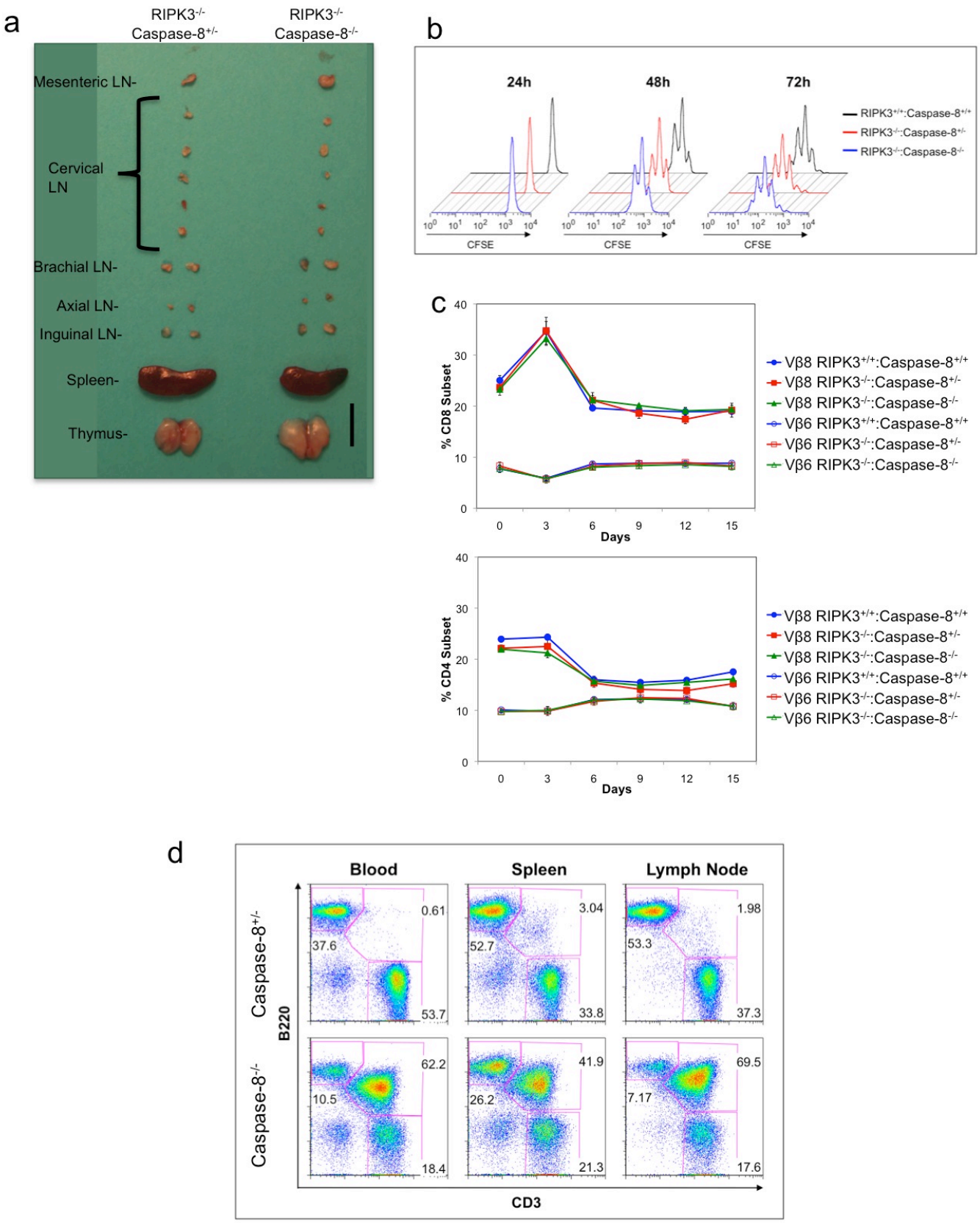




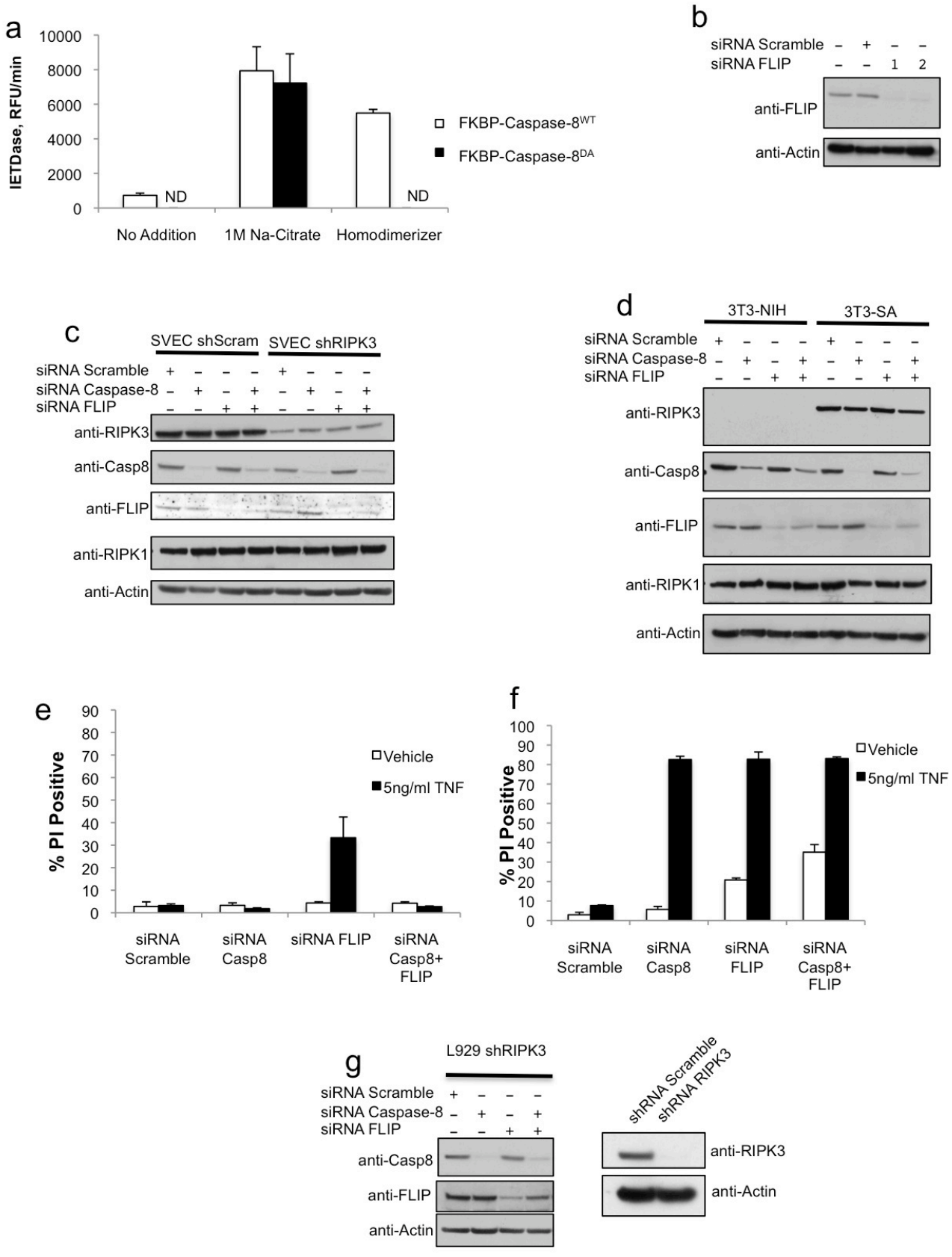




Oberst et al. Figure S2



Oberst et al. Figure S3



Oberst et al. Figure S4